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Resistance to malignant catarrhal fever in American bison (*Bison bison*) is associated with MHC class IIa polymorphisms

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Summary

The *Rhadinovirus* ovine herpesvirus-2 (OvHV-2) is the most common causative agent of malignant catarrhal fever (MCF) in clinically susceptible ruminants including cattle and bison. American bison (*Bison bison*) are highly susceptible to clinical MCF. Nevertheless, approximately 20% of bison on ranches or in feedlots become infected with the virus without developing clinical disease. Defining the genetic basis for differences in susceptibility between bison could facilitate development of improved control strategies for MCF. One genetic region that influences susceptibility to infectious diseases is the major histocompatibility complex (MHC). In this study, a *Bison bison* (*Bibi) DRB3* oligonucleotide microarray was used to type 189 bison from 10 herds where MCF outbreaks had occurred. Binary logistic regression was used to classify *DRB3* alleles as resistant (*R*), susceptible (*S*) or neutral (*N*). Animals were reclassified using six *DRB3* genotype categories: *N*/*N*, *N*/*R*, *N*/*S*, *R*/*S*, *R*/*R* and *S*/*S*. Analysis of homogeneity across herds showed that there was a herd effect. Consequently, a penalized logistic regression model was run with herd and genotype categories as the explanatory variables. The *R*/*R* genotype was associated with resistance to MCF ($P = 0.0327$), while the *S*/*S* genotype was associated with clinical MCF ($P = 0.0069$). This is the first evidence that MHC class IIa polymorphism is associated with resistance or susceptibility to OvHV-2-induced MCF.

Keywords *Bison bison*, disease association, major histocompatibility complex, major histocompatibility complex class II, malignant catarrhal fever.

Introduction

Malignant catarrhal fever (MCF) is an important disease for the commercial American bison (*Bison bison*) industry and is associated with significant economic losses (O'Toole *et al.* 2002; Li *et al.* 2006). Clinically affected bison generally develop vasculitis, and necrotizing lesions of mucosal surfaces including the oral cavity, trachea, esophagus, rumen, omasum, abomasum, jejunum and urinary bladder (Schultheiss *et al.* 1998; O'Toole *et al.* 2002). The lesions are characterized by lymphoid cell proliferation and infiltration (Schultheiss *et al.* 1998; O'Toole *et al.* 2002). Signs of clinical MCF in bison include lethargy, depression, wasting, nasal and ocular discharge and corneal opacity (O'Toole *et al.* 2002). All outbreaks in the United States have been associated with ovine herpesvirus-2 (OvHV-2), a *Rhadinovirus* from the gammaherpesvirus subfamily (O'Toole *et al.* 2002; Berezowski *et al.* 2005; Li *et al.* 2006). Sheep are the natural carriers of OvHV-2 and, therefore, serve as the reservoir for transmission to uninfected animals predominantly via aerosol transmission (Li *et al.* 2001a; 2004). There is currently no vaccine for OvHV-2. Separation of susceptible species from carrier hosts is the only way to prevent transmission.

While virtually all bison with clinical signs succumb to MCF, it is interesting that in wild and domesticated bison herds approximately 20% of animals seroconvert without progressing to clinical disease (Li *et al.* 1996; O'Toole *et al.* 2002; Zarnke *et al.* 2002; Berezowski *et al.* 2005). Genetic variation could influence either the host's ability to mount an effective immune response to the virus or alter the

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susceptibility of host cell populations to infection with OvHV-2 (Xu *et al.* 1993; Zanotti *et al.* 1996; Li *et al.* 1997; Lewin *et al.* 1999; Haan & Longnecker 2000; Schat & Davies 2000; Spear & Longnecker 2003). Because variation in the major histocompatibility complex (MHC) could affect either the immune response to OvHV-2 or viral attachment and entry, bison MHC class I and II genes are excellent candidate genes for MCF resistance.

Prior to this study, we characterized bison MHC class IIa haplotypes by DNA sequence analysis (Traul *et al.* 2005). Sequence analysis identified both previously described *Bison bison* (*Bibi*) *DRB3* alleles (Mikko *et al.* 1997), as well as new *Bibi-DRB3*, *Bibi-DQA* and *Bibi-DQB* alleles. The *Bibi-DRB3* sequences allowed us to reconfigure our existing *BoLA-DRB3* typing array for bison MHC typing by addition of bison specific probes (Park *et al.* 2004). To test the hypothesis that the bison MHC class IIa haplotypes affect susceptibility to MCF, this *BoLA/Bibi-DRB3* microarray was used to type bison with clinical MCF and antibody-positive bison without clinical MCF from herds with recent MCF outbreaks.

Materials and methods

Animals and samples

A total of 189 bison were included in this study. Seventy-seven of these bison had clinical MCF, and 112 were infected without clinical disease. Samples were obtained from several herds (Table 1), but were mainly from two feedlots in Kansas and Idaho, where there had been recent MCF outbreaks. All diagnoses of clinical MCF were confirmed by histopathology and nested or real-time PCR assays with OvHV-2 specific primers. The infected bison without clinical MCF were confirmed as antibody-positive via a competitive inhibition enzyme-linked immunosorbent assay (Li *et al.* 1994; 1995; 2001b). Some of these bison (≈ 10%) were also positive by OvHV-2-specific nested PCR. These animals survived in the feedlot for at least 5 months before being slaughtered. EDTA-blood and/or tissue samples were collected and used for the isolation of DNA for MHC typing. DNA was extracted from buffy coats or tissues by phenol-chloroform extraction, ethanol precipitated, quantified by spectroscopy and stored at −20 °C (Maniatis *et al.* 1982). All experiments complied with US laws pertaining to animal welfare and safe laboratory practices.

Table 1 Sources of bison samples.

Location	Animals	MCF+[1]	MCF−[2]
Scott City, KS, USA	98	16	82
Twin Falls, ID, USA	43	25	18
Laramie, WY, USA	13	1	12
Akron, CO, USA	16	16	0
Fort Collins, CO, USA	10	10	0
Chippewa Falls, WI, USA	2	2	0
San Bernadino, CA, USA	3	3	0
Sulfer, OK, USA	1	1	0
Ames, IA, USA	1	1	0
Westaskwin, AB, Canada	2	2	0
Total	189	77	112

MCF, malignant catarrhal fever.
[1]Bison classified as infected with disease (MCF+) had clinical signs of MCF, tested positive for ovine herpesvirus-2 DNA by either nested or real-time PCR, and the diagnosis was confirmed by histopathology.
[2]Bison classified as infected without disease (MCF−) were seropositive for MCF viral antibody by competitive inhibition enzyme-linked immunosorbent assay and survived for 5 months or more without developing clinical disease.

Microarray-based *DRB3* typing

The *DRB3* typing array was based on 66 *BoLA-DRB3* and 15 *Bibi-DRB3* exon 2 sequences (Mikko *et al.* 1997; Traul *et al.* 2005). The array comprised five series of exon 2 probes (15 for codons 8–15, 24 for codons 27–33, 18 for codons 54–61, 28 for codons 66–72 and 12 for codons 73–79), and included 10 bison specific probes. Major histocompatibility complex typing was performed as previously described (Park *et al.* 2004). Briefly, arrays comprised 15–22 base oligonucleotide probes (Invitrogen) spotted on epoxy-silane coated, 12-well, Teflon-masked glass slides (Tekdon, Inc.) using a Microgrid II BioRobotics arrayer (Genomic Solutions; Call *et al.* 2001). Genomic *Bibi-DRB3* exon 2 targets for hybridization to arrays were generated by PCR with biotinylated primers (BoDRB3FP-HL030 and BoDRB3RP-HL032; van Eijk *et al.* 1992). Following PCR amplification, 12 μl of reaction mix was diluted to 79 μl in hybridization buffer and hybridized to the array overnight at 50 °C. Slides were scanned with an ArrayWoRx scanner (Applied Precision). Microarray hybridization spots were subjectively scored on a 5-point scale from negative to strongly positive. Proprietary CYTOFILE genotyping software (available for C. J. Davies) was used to sort the data matrix with a pre-defined allele definition index (Davies 1988; Davies *et al.* 1994).

MHC class II sequencing

Sequencing was used to identify the class II alleles carried by bison with equivocal *DRB3* typing results ($n = 6$). Sequencing of bison *DRB3*, *DQA* and *DQB* alleles was performed as previously described (Traul *et al.* 2005) with a minor modification of using two new multiplexed *DQB* reverse primers (BoDQB-RP-E2H, 5′-GCACTCACCTAGCCGCTGCCA-3′ and BoDQB-RP-E2I, 5′-GCACTCACCTAGCCGCTGCAA-3′).

Statistical analysis

Phenotypic frequencies (f) were calculated using the formula $f =$ (number of bison with allele/total number of

bison) × 100. Allele frequencies (g) were calculated using the formula

$$g = \frac{\begin{array}{l}(\text{number of bison with allele} \\ \quad + \text{number of homozygotes}) \\ \quad /\text{total number of bison}\end{array}}{2} \times 100$$

Data analysis was performed in four stages. First, binary logistic regression models were run for each of the 18 alleles with clinical disease as the response variable: no clinical disease ($y = 0$) or clinical MCF ($y = 1$). *DRB3* alleles were classified as neutral (N), susceptible (S) or resistant (R) based on their P-value and Z-statistic; alleles with P-values ≤ 0.30 were classified as either resistant or susceptible based on the sign of the Z-statistic (Hosmer & Lemeshow 2000), the remaining alleles were classified as neutral. Animals were assigned to six genotype classes based on the classification of their two alleles: *neutral*/*neutral* (N/N), *neutral*/*resistant* (N/R), *neutral*/*susceptible* (N/S), *resistant*/*susceptible* (R/S), *resistant*/*resistant* (R/R) and *susceptible*/*susceptible* (S/S). This was followed with a Chi-squared test of homogeneity to see if there was a difference in genotype frequencies across herds. Finally, a penalized logistic regression model (Firth 1993) was run with herd and genotype categories as the explanatory variables and presence or absence of clinical disease as the response variable. Our initial model included a herd × genotype class interaction term. However, this term had a P-value of 0.92 and, therefore, was removed from the final overall model. All analyses were performed using SAS®/STAT software (SAS® Institute). Results with P-values ≤ 0.05 were considered statistically significant.

Results

Microarray-based *Bibi-DRB3* typing

The *DRB3* typing array included probes defining 15 *Bibi-DRB3* alleles. The array was validated using 14 bison from our previous sequencing study (Figure S1; Traul *et al.* 2005). In the current study, 17 *Bibi-DRB3* alleles and one *BoLA-DRB3* allele were found in the 189 bison. This included 12 *Bibi-DRB3* alleles identified in our previous study (Traul *et al.* 2005), as well as two *Bibi-DRB3* alleles (*DRB3*0102* and *DRB3*0601*; GenBank accession nos. DQ353803 and DQ353804 respectively) previously described by Mikko *et al.* (1997). Class II alleles of six animals that could not be definitively typed with the microarray were sequenced. These animals contained three new *Bibi-DRB3* alleles (*DRB3*0702*, *DRB3*1101* and *DRB3*1201*; GenBank accession nos. DQ353805–DQ353807 respectively) and one cattle allele (*BoLA-DRB3*1101*; Table 2). The *DRB3*0501* allele described by Mikko *et al.* (1997) was not carried by any of the bison in this study. Phenotypic frequencies of *Bibi-DRB3* alleles ranged from 0.5% to 33.3% in these 189 bison.

Analysis of associations between *Bibi-DRB3* alleles and MCF disease status

To study the association between MCF status and *DRB3* alleles, we ran one-at-a-time binary logistic regression using the number of copies for a specific allele as a predictor and the absence (MCF−) or presence (MCF+) of disease as the response variable. Eighteen models were run (Table 2). One allele, *DRB3*0801*, was significantly associated with resistance to MCF ($P = 0.005$). Notably, all five *DRB3*0801* homozygotes were in the MCF− group (Table 2; Fisher's exact $P = 0.02$). Another allele, *DRB3*0602*, was significantly associated with clinical disease ($P = 0.048$). Furthermore, there were six *DRB3*0602* homozygotes in the MCF+ group vs. only one homozygote in the MCF− group (Table 2; Fisher's exact $P = 0.03$).

The *DRB3* alleles were ranked from most significant (smallest P-value) to least significant (largest P-value) and, based on the sign of the Z-statistic, assigned to three categories: susceptible (S; positive slope, $P \leq 0.30$), resistant (R; negative slope, $P \leq 0.30$) and neutral (N; $P > 0.30$ irrespective of sign). The $P \leq 0.30$ significance level followed the $P \leq 0.25$ significance level used by Hosmer & Lemeshow (2000) for univariable models. Because *DRB3*1101*, *DRB3*1201* and *BoLA-DRB3*1101* were carried by only 3, 1 and 2 bison respectively, they were assigned to the neutral category. Based on their *DRB3* genotype, bison were classified as N/N, N/R, N/S, R/S, R/R or S/S. To assess if animals in the six genotype categories were distributed homogenously across herds, we conducted a Chi-squared test for homogeneity across three groups: the Scott City herd with 98 bison, the Twin Falls herd with 43 bison and the remaining 48 bison (Table 3). Genotype frequencies were not homogenously distributed across herds ($P = 0.008$), which was expected because numbers of diseased and clinically normal bison were not evenly distributed across herds (Table 1). Therefore, herd effect was included in the final model.

Because all 14 of the bison in the R/R category were in the clinically normal (MCF−) group, a maximum likelihood could not be estimated. Therefore, a penalized logistic regression model (Firth 1993) was used (Table 4). The logistic regression model had a per cent concordance of 76.9% and the Hosmer and Lemeshow goodness-of-fit statistics indicated that the data fit the model well. Animals in the R/R category, having allele *DRB3*0801* with *DRB3*0801* or *DRB3*0501*, were resistant to MCF ($P = 0.0327$). The P-value for the R/R genotype was relatively high compared with the univariate P-value for *DRB3*0801* from Table 2 of 0.005 because of the penalties associated with complete separation of data. Complete separation makes SE very large so the SE for the R/R genotype was 1.24, which was substantially more than the next largest standard error of 0.57 (Table 4). Bison in the S/S category, having alleles *DRB3*0602*, *DRB3*0102* and *DRB3*0101*, were associated with clinical MCF ($P = 0.0069$). As

Table 2 Comparison of *DRB3* allele frequencies in bison exposed to OvHV-2.

	Number of bison		Number of homozygotes		Allele frequency[1]					
DRB3 allele	MCF+	MCF−	MCF+	MCF−	All	MCF+	MCF−	*Z*-statistic[2]	*P*-value	Allele class[3]
**0101*	9	9	1	0	0.050	0.065	0.040	1.04	0.298	*S*
**0102*	9	7	2	1	0.050	0.071	0.036	1.34	0.179	*S*
**0103*	6	9	1	0	0.042	0.045	0.040	0.24	0.810	*N*
**0201*	16	21	2	2	0.108	0.117	0.103	0.42	0.677	*N*
**0301*	10	14	0	2	0.069	0.065	0.071	−0.23	0.815	*N*
**0401*	6	7	0	0	0.034	0.039	0.031	0.41	0.681	*N*
**0502*	14	29	0	0	0.114	0.091	0.129	−1.24	0.216	*R*
**0601*	6	5	0	0	0.029	0.039	0.022	0.95	0.343	*N*
**0602*	30	33	6	1	0.185	0.234	0.152	1.97	0.048	*S*
**0701*	16	22	0	0	0.101	0.104	0.098	0.19	0.848	*N*
**0702*	1	2	0	0	0.008	0.006	0.009	−0.26	0.793	*N*
**0801*	6	27	0	5	0.101	0.039	0.143	−2.82	0.005	*R*
**0802*	3	6	0	0	0.024	0.019	0.027	−0.46	0.644	*N*
**0901*	6	9	0	0	0.040	0.039	0.040	−0.06	0.951	*N*
**1001*	2	7	1	1	0.029	0.019	0.036	−0.78	0.435	*N*
**1101*	0	3	0	0	0.008	0.000	0.013	–	–	*N*
**1201*	1	0	0	0	0.003	0.006	0.000	–	–	*N*
BoLA[4]	0	2	0	0	0.005	0.000	0.009	–	–	*N*
Total	77	112	13	12	1.00	1.00	1.00			

MCF, malignant catarrhal fever; OvHV-2, ovine herpesvirus-2.

[1]Frequencies calculated by allele counting.

[2]Logistic regression *Z*-statistic. A positive number indicates an association with MCF+ and a negative number with MCF−.

[3]*DRB3* alleles were classified as neutral (*N*), susceptible (*S*) or resistant (*R*) based on their *Z*-statistic and *P*-value. Alleles with *P*-values ≤ 0.30 were classified as either resistant or susceptible and the remaining alleles were classified as neutral.

[4]Cattle allele *BoLA-DRB3*1101* (Davies *et al.* 1997; Russell *et al.* 1997). These bison carried the *BoLA-DH22F* haplotype comprised *BoLA-DRB3*1101*, *BoLA-DQA1*0102* and *BoLA-DQB*0402* (Davies *et al.* 1994; Park *et al.* 2004).

expected, the Scott City herd, the only herd with more MCF− than MCF+ bison, had a different distribution of *DRB3* genotypes from the other groups ($P < 0.0001$).

Discussion

In this study we defined MCF susceptibility (MCF+) as the development of clinical disease, which is almost always fatal. Malignant catarrhal fever resistance (MCF−) was defined as infection (seroconversion to OvHV-2) without the development of clinical disease. By including only seropositive bison in the disease-resistant group, we were sure that all of the bison in this group were infected with OvHV-2. Nevertheless, it is likely that individual bison were exposed to different amounts of virus. It is well established that high-dose exposure to a pathogen can overwhelm the immune system even in the most resistant animals. Sheep are carriers of OvHV-2 and never develop clinical MCF under natural flock conditions, but it is possible to experimentally induce clinical disease in sheep by intranasal inoculation with a very high dose of virus (Li *et al.* 2005). The Twin Falls, Idaho bison in our study were exposed to a very high dose of OvHV-2. In the Twin Falls outbreak a flock of 1375 7-month-old lambs and 375 ewes were grazed within 600 yards of the bison feedlot and 825 out of 1610 bison died of MCF (51.2% mortality; Li *et al.* 2006). Only six bison in the current study with the *DRB3*0801* allele, associated with resistance, had clinical MCF and five of these bison were from the Twin Falls herd.

We found statistically significant associations between MCF resistance/susceptibility and *Bibi-DRB3* alleles. In a previous study we found that susceptibility to *Staphylococcus aureus* mastitis in cattle was associated with MHC haplotypes with non-duplicated *BoLA-DQ* genes (Davies *et al.* 1994; Park *et al.* 2004). However, comparison of bison MHC class IIa haplotypes did not reveal an association between duplicated *DQ* genes and MCF resistance or susceptibility (data not shown). Furthermore, the *DRB3*0802* and *DRB3*0901* alleles, which were linked to the same *Bibi-DQA* and *Bibi-DQB* alleles as *DRB3*0801*, were not associated with MCF resistance (Table 2; Traul *et al.* 2005).

Several studies have shown that MHC class I-restricted CD8+ cytotoxic T lymphocytes are required to control acute gammaherpesvirus diseases, including the initial lytic phase of infection with either EBV or MHV68 (Callan 2004; Stevenson & Efstathiou 2005). Major histocompatibility complex class I molecules may play a similar critical role in control of OvHV-2 infection in bison during the initial infection. Consequently,

Table 3 Number of bison classified by *DRB3* genotype and disease status[1].

Disease status	Herd	*N/N* bison	*N/R* bison	*N/S* bison	*R/S* bison	*R/R* bison	*S/S* bison	All bison
MCF–[2]	Scott City	27 (24)	12 (13)	21 (21)	9 (11)	11 (10)	2 (3)	82
	Twin Falls	4 (5)	5 (3)	3 (5)	4 (2)	1 (2)	1 (1)	18
	Other	2 (2)	1 (2)	4 (3)	2 (2)	2 (2)	1 (0)	12
	All herds[5]	33 (32)	18 (18)	28 (30)	15 (13)	14 (8)	4 (11)	112
MCF+[3]	Scott City	5 (4)	1 (3)	7 (5)	1 (1)	0 (0)	2 (3)	16
	Twin Falls	4 (7)	7 (4)	4 (7)	5 (2)	0 (0)	5 (5)	25
	Other	12 (10)	5 (6)	11 (10)	1 (3)	0 (0)	7 (7)	36
	All herds[5]	21 (22)	13 (13)	22 (20)	7 (9)	0 (6)	14 (7)	77
All bison[4]	Scott City	32 (28)	13 (16)	28 (26)	10 (11)	11 (7)	4 (9)	98
	Twin Falls	8 (12)	12 (7)	7 (11)	9 (5)	1 (3)	6 (4)	43
	Other	14 (14)	6 (8)	15 (13)	3 (6)	2 (4)	8 (5)	48
	All herds	54	31	50	22	14	18	189

MCF, malignant catarrhal fever.

[1]Actual and (expected) number of bison in each class, with expected number of bison estimated by the chi-squared test for homogeneity. Genotype classes were *neutral/neutral* (*N/N*), *neutral/resistant* (*N/R*), *neutral/susceptible* (*N/S*), *resistant/susceptible* (*R/S*), *resistant/resistant* (*R/R*) and *susceptible/susceptible* (*S/S*). Disease groups: clinical MCF (MCF+), infected without clinical disease (MCF–) and all bison regardless of disease status.

[2]Chi-squared test for homogeneity across herds indicated that *DRB3* genotypes of MCF– bison were evenly distributed ($P = 0.64$).

[3]Chi-squared test for homogeneity across herds without the *R/R* category, which did not include any bison, indicated that *DRB3* genotypes of MCF+ bison were evenly distributed ($P = 0.11$).

[4]Chi-squared test for homogeneity across herd indicated that *DRB3* genotypes of all bison were not evenly distributed ($P = 0.008$). Because the numbers of diseased and clinically normal bison were not evenly distributed across herds, this was the expected result.

[5]Chi-squared test for homogeneity across disease groups indicated that *DRB3* genotypes were not evenly distributed ($P = 0.0008$).

sequence-based characterization of bison MHC class I haplotypes and evaluation of associations between class I haplotypes and resistance to OvHV-2 are high priorities. Furthermore, it is imperative that the associations identified in the present study be confirmed in challenge studies.

Table 4 Penalized logistic regression analysis of genotype and herd effects.

Effect[1]	Parameter estimate	SE	Wald Chi-squared test	*P*-value
Intercept	–0.42	0.29	2.06	0.1514
N/R genotype	0.25	0.45	0.32	0.5711
N/S genotype	0.58	0.40	2.06	0.1509
R/S genotype	–0.14	0.50	0.08	0.7747
R/R genotype[2]	–2.64	1.24	4.56	0.0327
S/S genotype[2]	1.54	0.57	7.29	0.0069
Scott City herd[3]	–1.47	0.24	36.81	<0.0001
Twin Falls herd	0.38	0.27	2.04	0.1530

[1]The *N/N* genotype and the 'other herd' categories were used as references. The probability of the null hypothesis for the overall model was $P < 0.0001$.

[2]The *R/R* and *S/S* genotypes had significant effects on disease susceptibility. All bison with the *R/R* genotype were in the MCF– group (Table 3). This separation of the data for the *R/R* genotype resulted in a large 'penalty'; therefore, the *P*-value was relatively high ($P = 0.0327$).

[3]The Scott City herd was significantly associated with disease resistance. This was not surprising as the majority (73%) of the clinically normal animals were from this herd.

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Supplementary Material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01575.x

Figure S1 Example of microarray typing data.

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